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Proline-Rich Tyrosine Kinase 2 Mediates Gonadotropin-Releasing Hormone Signaling to a Specific Extracellularly Regulated Kinase-Sensitive Transcriptional Locus in the Luteinizing Hormone β -Subunit Gene

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Abstract

G protein-coupled receptor regulation of gene transcription primarily occurs through the phosphorylation of transcription factors by MAPKs. This requires transduction of an activating signal via scaffold proteins that can ultimately determine the outcome by binding signaling kinases and adapter proteins with effects on the target transcription factor and locus of activation. By investigating these mechanisms, we have elucidated how pituitary gonadotrope cells decode an input GnRH signal into coherent transcriptional output from the LH β -subunit gene promoter. We show that GnRH activates c-Src and multiple members of the MAPK family, c-Jun NH₂-terminal kinase 1/2, p38MAPK, and ERK1/2. Using dominant-negative point mutations and chemical inhibitors, we identified that calcium-dependent proline-rich tyrosine kinase 2 specifically acts as a scaffold for a focal adhesion/cytoskeleton-dependent complex comprised of c-Src, Grb2, and mSos that translocates an ERK-activating signal to the nucleus. The locus of action of ERK was specifically mapped to early growth response-1 (Egr-1) DNA binding sites within the LH β -subunit gene proximal promoter, which was also activated by p38MAPK, but not c-Jun NH₂-terminal kinase 1/2. Egr-1 was confirmed as the transcription factor target of ERK and p38MAPK by blockade of protein expression, transcriptional activity, and DNA binding. We have identified a novel GnRH-activated proline-rich tyrosine kinase 2-dependent ERK-mediated signal transduction pathway that specifically regulates Egr-1 activation of the LH β -subunit proximal gene promoter, and thus provide insight into the molecular mechanisms required for differential regulation of gonadotropin gene expression.

THE MAPKS ARE A FAMILY of evolutionarily conserved serine/threonine kinases that transmit externally derived signals regulating cell growth, division, gene transcription, differentiation, and apoptosis (1). There are a plethora of mechanisms by which G protein-coupled receptors (GPCRs) activate MAPKs; this underpins the fundamental nature of this receptor-mediated effect in cell physiology (for review see Refs. 2 and 3). GPCR activation of several types of G proteins (*Gas*, *Gao*, *Gaq/11*, *Gai*) can initiate ERK (a MAPK isoform) activation, yet there is a radiation of the signal transduction process distal to G protein activation

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(for review see Ref. 4). Signals subsequently generated by second messenger-dependent protein kinases [protein kinase A and protein kinase C (PKC) (5)], cross talk-activated epidermal growth factor receptor (EGFR) (6) or heterodimerized integrin receptors [involving focal adhesion kinase (FAK); or proline-rich tyrosine kinase 2 (Pyk2) activation; (7)] all eventually converge at the level of the canonical ERK-activating cascade (4). We and others have demonstrated that generic GPCR-mediated ERK activation is reliant upon the formation of complex multiprotein superstructures (8-11), and insights have recently been generated with respect to the nature of the catalytic activity of ERK depending upon which specific proteins constitute the ERK-activating superstructure (4,12).

The GPCR, GnRH receptor (GnRHR), controls the differential synthesis and release of LH and FSH from bihormonal anterior pituitary gonadotrope cells, by binding the hypothalamic peptide, GnRH. Stimulation of the GnRHR triggers an intracellular second messenger-signaling cascade that increases gene expression of the common α -glycoprotein subunit and hormone-specific LH and FSH β -subunits primarily via activation of different MAPK isoforms (13-19). GnRHR-mediated activation of ERK is dependent upon the functionality of focal adhesion-linked proteins (9) and highlights that several distinct mechanisms of ERK activation can exist because GnRH-mediated ERK activation can also occur through activation of PKC (5) and reportedly through an EGFR-cross talk mechanism (20). The identities of the key scaffold proteins required for GnRH-induced MAPK activation are unknown, but there is increasing evidence that protein scaffolds and cross talk between different signaling pathways do control the outcome of GnRHR signaling (21,22).

Instead, there has been considerable focus on gonadotropin gene promoter regulatory DNA elements required for pituitary-specific basal expression, especially how these combine and synergize with GnRH-regulatory DNA elements (reviewed in Ref. 23). Briefly, the LH β -subunit gene promoter encodes GnRH-activating regions comprised of DNA binding sites for homeodomain factors (Pitx1 and Otx-related), nuclear receptor steroidogenic factor-1 (SF-1), Sp1, and GnRH-induced early growth response-1 (Egr-1), which form an activating complex (Ref. 24 and references therein). These factors are obligate for LH β -subunit gene expression (25-27) and may also be regulated by GnRH (28-30) by as yet undefined mechanisms. Indeed, the link between GnRH induction of MAPK isoforms, multiprotein activating complex, and the transcriptional target that combine to subsequently activate LH β -subunit gene expression has not been elucidated.

Here we report, using the α -glycoprotein subunit-expressing α T3-1 (31) and LH-expressing L β T2 (32) gonadotrope cell lines, a novel GnRH-induced, Pyk2-mediated, ERK-signaling pathway that is conserved between these cell lines. We demonstrate GnRH activation of this pathway, other MAPK family members [p38MAPK and c-Jun NH₂-terminal kinase 1/2 (JNK)] and the soluble tyrosine kinase c-Src, and map these to distinct GnRH-activation sites on the LH β -subunit gene promoter. A direct link between Egr-1 transcriptional activation, ERK and p38MAPK signaling, and regulation of promoter activity was established. This advances the understanding of how the GnRH signal is transduced from the plasma membrane to the nucleus, identifies Pyk2 as an ERK-scaffold protein important for mediating GnRH regulation of LH β -subunit gene expression, and elucidates how GnRH-MAPK signaling regimes are decoded into gene expression via Egr-1.

RESULTS

GnRH-Induced MAPK Activation in Gonadotrope Cells

Adding a physiological dose of 10 nM GnRH to α T3-1 or L β T2 cells rapidly elevated the levels of active phosphorylated ERK1/2. Activation was measured at 1 min and could still be measured at 90 min (Fig. 1, A-C). Because this prolonged activation is atypical of GPCRs, the

stimulation of c-Src and other MAPK family members by GnRH was investigated and compared with ERK1/2. GnRH elevated levels of active-JNK1/2, active-p38MAPK, and c-Src (Fig. 2, A-D). Comparison of panels A-D indicated that all active-MAPKs and c-Src were measurable by 5 min. Given that active-ERK1/2 rapidly responded to GnRH stimulation and that ERK activation has been extensively studied in GPCR signaling, we focused on the mode of activation of ERK in the context of gonadotrope cell lines.

Putative Roles of EGFR, Ca^{2+} , and Cytoskeleton in GnRH-Induced ERK1/2 Activation

The stimulation of MAPK isoforms by many GPCRs has been reported to occur via an epidermal growth factor receptor (EGFR) transactivation mechanism (33). GnRH-mediated ERK1/2 activation in the two gonadotrope cell lines was resistant to chemical inhibition of the EGFR intrinsic tyrosine kinase with AG1478 pretreatment (Fig. 3). However in both $\alpha\text{T3-1}$ and $\text{L}\beta\text{T2}$, EGF (10 ng/ml, 10 min)-mediated tyrosine phosphorylation of the EGFR and subsequent activation of ERK1/2 was abrogated by AG1478 pretreatment (Fig. 3). In addition, it was evident that there was no significant GnRH-induced increase in EGFR tyrosine phosphorylation. We next assessed the GnRH-induced activation of ERK1/2 in both $\alpha\text{T3-1}$ and $\text{L}\beta\text{T2}$ cells in the presence of chemical agents that have been demonstrated to inhibit other GPCR-mediated ERK1/2 activation signals. As demonstrated in Fig. 4 GnRH-induced ERK1/2 activation was sensitive to chelation of intracellular calcium release by 1,2-bis (2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid acetoxymethyl ester (BAPTA-AM) and to inhibition of PKC with both depletion of endogenous PKC by phorbol12-myristate-13-acetate chronic exposure and by inhibition of PKC activity by Ro-31-8220 preincubation. GnRH-induced ERK1/2 activation was also sensitive to disruption of active cytoskeletal dynamics with cytochalasin-D and latrunculin-B. Using a panel of tyrosine kinase inhibitors, it was demonstrated that although resistant to inhibition of the EGFR kinase, the GnRH-induced ERK1/2 activation is sensitive to less specific tyrosine kinase inhibitors, herbimycin-A and c-Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(t -butyl)pyrazolo[3,4- d]pyrimidine (PP2). The lack of effect of 10 nM wortmannin demonstrates that the GnRH-induced ERK1/2 activation was independent of the kinase activity of phosphatidylinositol 3-kinase. Furthermore, using a 100–200 nM concentration of wortmannin has no effect on LH β -subunit gene expression or activation of ERK [data not shown; (13)]. Considering the novel lack of EGFR-transactivation dependence of the GnRH-induced ERK1/2 activation and its sensitivity to general tyrosine kinase inhibition, we next investigated in an unbiased manner the general nature of control of tyrosine phosphorylation in gonadotrope cells upon GnRH stimulation.

GnRH-Mediated Tyrosine Phosphorylation in $\alpha\text{T3-1}$ and $\text{L}\beta\text{T2}$ Gonadotropes

GnRH stimulation of both the gonadotrope cell lines resulted in the increase in phosphotyrosine status of several proteins (Fig. 5, panel A, $\alpha\text{T3-1}$; panel B, $\text{L}\beta\text{T2}$). The major protein tyrosine phosphorylated in response to GnRH was approximately 115 kDa. Using a specific antiserum to Pyk2, we determined that the approximately 115-kDa protein was indeed the Ca^{2+} -dependent tyrosine kinase also known as FAK2 or RAFTK. Upon GnRH stimulation there was a rapid and relatively long-lasting increase in the tyrosine phosphorylation status of specifically immunoprecipitated Pyk2 (Fig. 5, C-D). We found that expression of the other main focal adhesion kinase, FAK, was minimal and demonstrated only a minimal elevation of its tyrosine phosphorylation status (data not shown). We next assessed whether there was a commonality in Pyk2/ERK1/2 signal sensitivity to our panel of chemical inhibitors. We were specifically interested in the ERK1/2 signal generation in $\text{L}\beta\text{T2}$ cells as they can functionally synthesize mature LH in an ERK-dependent manner (13). Compared with the sensitivity to inhibitors of c-Src, PKC, and EGFR kinase activity, the GnRH-mediated Pyk2 stimulation displayed a similar pattern to that for ERK1/2 activation, suggesting that the two events may be physically associated (Fig. 5E). A profound inhibition of Pyk2 activation was also observed after preincubation with cytochalasin-D and latrunculin-B, further reinforcing the role of focal

adhesions in the GnRH-mediated ERK1/2 activation. We therefore treated the cells with Arg-Gly-Asp-Ser (RGDS) tetrapeptides, which have been used extensively to disrupt cytoskeletal organization in cellular tissues via their capacity to disrupt focal adhesion complexes (7,9). Preincubation of L β T2 cells with RGDS (1 mM) peptide inhibited the GnRH-induced activation of both Pyk2 and ERK1/2 (Fig. 6). A similar preincubation with Arg-Gly-Glu-Ser (RGES) tetrapeptide had no significant effect upon GnRH action. Neither RGDS nor RGES tetrapeptide had any significant effect upon EGF (10 ng/ml, 10 min)-stimulated ERK1/2 activation.

GnRH-Induced Signaling Protein Complex Assembly at Pyk2

Because we demonstrated a c-Src dependence involved in GnRHR-mediated Pyk2 and ERK1/2 activation, we investigated whether there was signal complex assembly between these two proteins. GnRH stimulation elevated the relative c-Src content of Pyk2 immunoprecipitates (Fig. 7). The association was persistent, lasting up to 1 h after GnRH stimulation. Preincubation with the cytoskeletal disruptors, cytochalasin-D and latrunculin-B, and the tyrosine kinase inhibitor herbimycin-A all disrupted the ability of GnRH to induce signaling complex assembly between c-Src and Pyk2. In our experimental paradigm we propose that ERK-activating complex assembly occurs at the Ca²⁺-dependent tyrosine kinase Pyk2 and not at the EGFR; therefore we demonstrated that GnRH stimulation induced association of Pyk2 with the mSos binding protein Grb2. GnRH stimulation induced a time-dependent elevation in the relative Grb2 content of Pyk2 immunoprecipitates (Fig. 7C), and in the reciprocal experiment the relative Pyk2 content of Grb2 immunoprecipitates also increased (Fig. 7D). We also noted that the Pyk2 associated with the Grb2 immunoprecipitates was tyrosine phosphorylated in response to GnRH stimulation (Fig. 7E).

GnRH-Induced Signaling Complex Assembly and ERK1/2 Activation

To demonstrate the role of Pyk2 in ERK activation, we employed point mutants of Pyk2 and observed their effect on GnRH activation of a cotransfected myc-tagged ERK2. Overexpression of a kinase inactive Pyk2 mutant (K475A) abrogated the GnRH-induced activation of the cotransfected myc-tagged ERK2 (Fig. 8). Overexpression of the Y402F mutant of Pyk2, in which the primary c-Src interacting site is lost, also attenuated the capacity of GnRH to activate the myc-tagged ERK2. Overexpression of the Y881F mutant of Pyk2, in which the major Grb2 binding site tyrosine is also mutated, led to a reduction in the ability of GnRH to activate myc-tagged ERK2. Thus it appears that a productive activation of the Pyk2 intrinsic tyrosine kinase and subsequent protein-protein association with c-Src and Grb2 are required for GnRH-induced ERK activation. Because we have demonstrated GnRH induces a Grb2 translocation to tyrosine-phosphorylated Pyk2 we next investigated whether the ERK1/2 activation pathway was Ras dependent. Cotransfecting the myc-tagged ERK2 reporter with a dominant-negative form of Ras (N17Ras) inhibited GnRH-mediated ERK2 activation (Fig. 8). Overexpression of a dominant-negative c-Raf (Raf-C4B), the direct downstream effector of Ras, also inhibited GnRH-induced ERK2 activation. Preincubation of L β T2 cells with the MAPK kinase (MEK1/2) inhibitor, PD98059, demonstrated that the eventual activation of ERK1/2 is due to the dual tyrosine-threonine kinase MEK1/2.

GnRH-Induced Nuclear Activation of ERK1/2

L β T2 cells stained for the active form of ERK1/2, in the absence of GnRH agonist in serum-starvation conditions, demonstrated minimal staining (Fig. 9, panel A, images 1 and 2). Application of GnRH (10 nM) induced an elevation in the background cytoplasmic staining and an increase in the appearance of active ERK1/2 in the cell nucleus. With greater time periods of GnRH stimulation, there was a significant increase in the amount of active ERK1/2 staining across the entire nucleus but not in the nucleolus (Fig. 9A, images 3-8). Nuclear staining for active ERK1/2 persisted for up to 120 min and with greater cellular magnification it was noted

that active ERK1/2 regions became annular in structure within the nucleus (data not shown). The degree of nuclear active ERK1/2 was significantly attenuated by pretreatment of the cells with cytochalasin-D or latrunculin-B (Fig. 9B, images 5-8) indicating the important role of focal adhesions in GnRH-mediated ERK1/2 activation.

Mapping of GnRH Response Elements in the LH β -Subunit Gene Promoter

Having defined GnRH induction and translocation of active ERK1/2, we then examined possible regulatory targets of this nuclear signaling complex. The -1786 bp of the ovine LH β -subunit gene promoter is regulated by GnRH *in vivo* (34); therefore we assayed this element for GnRH responsiveness *in vitro* using L β T2 gonadotrope cells (Fig. 10, panel A). To map the GnRH-responsive regions within this promoter fragment, 5'-deletions were generated and tested using transient transfection assays. This analysis identified two distinct loci (Fig. 10B), a distal region mapped between -692 to -626 bp, which was named the upstream response region (URR), and a proximal promoter (PP) region mapped within -320 bp of the transcription start site. The intervening DNA region between -626 and -320 may contain a negative regulatory *cis*-acting DNA element, because successive 5'-deletions appeared to restore GnRH responsiveness to the PP. To further test whether the URR and PP were independently GnRH responsive, another series of 5'-deletion constructs were engineered upstream of a luciferase reporter. Because luciferase reporter assays are more sensitive than β -galactosidase, small changes in reporter activity can be easily measured. These constructs were transiently transfected and treated with GnRH (Fig. 10C). Deletion of the PP, but retaining the URR (URR Δ PP1; -692 to -322 bp) and the proposed negative regulatory DNA sequence, failed to produce a significant GnRH response, but deletion of the DNA sequence between -630 and -322 bp (URR Δ PP2; -692 to -630 bp) restored GnRH responsiveness to the URR. Further deletion of the -320 -bp fragment, by removing an additional 183 bp localized the PP to within -136 bp. This short DNA fragment contains DNA binding and transactivation sites for SF-1 and Pitx1 (35,36) and encodes two putative Egr-1 DNA binding sites (37). Deletion of the -136 -bp region to -29 bp abolished GnRH-induced activity, confirming the importance of the encoded transcription factor binding sites. Together, these data indicate that the URR and PP are independent GnRH-response regions separated by a negative *cis*-acting regulatory element.

Regulation of the LH β -Subunit Gene Promoter by MAPK

To determine which GnRH-induced MAPK signaling response was required for function of the URR and PP, we selected three constructs encoding -692 , -320 , and -136 bp and compared responses after GnRH stimulation and after selective blockade of the various MAPK signaling cascades (Fig. 11, A-D). Treatment with the c-Src inhibitor PP2 reduced promoter activity by 50% (Fig. 11A). Pretreatment with the specific MEK1/2 inhibitor PD98059 or cotransfection of a dominant-negative MEK (dnMEK) plasmid indicated that the GnRH-induced ERK-signaling response occurred via the PP (Fig. 11, B and D). Inhibition of JNK/p38MAPK using high doses of SB203580 significantly repressed output across the promoter (Fig. 11B), but addition of a different JNK/p38MAPK inhibitor SB202190, at lower doses ($1-10 \mu\text{M}$) blocked the PP (Fig. 11C). The JNK/p38MAPK inhibitors, SB203580 and SB202190, can inhibit both JNK and p38MAPK at high doses, but only inhibit p38MAPK at low doses (38). Cotreatment with PD98059 and SB203580 significantly repressed output from the -320 and -136 bp promoters when compared with PD98059, indicating that MEK1/2 and p38MAPK signaling converged on the PP. Collectively, we deduced that the -692 -bp promoter encoding the URR was sensitive to high doses of JNK/p38MAPK inhibitor, suggesting that JNK signaling was important, and that the PP was sensitive to blockade of c-Src, p38MAPK, and ERK.

MAPK Regulation of Egr-1 Expression and DNA Binding

The studies indicate that the PP contains important regulatory elements that mediate GnRH-dependent activation via the ERK and p38MAPK pathways. Egr-1 protein is a dynamic effector of GnRH stimulation of the LH β -subunit gene promoter, is highly regulated by GnRH acting via PKC and ERK in α T3-1 cells, and is required for LH β -subunit gene expression *in vivo* (25,39-43). To examine whether Egr-1 was the actual target of the MAPK-regulated GnRH-response in L β T2 cells, we assayed Egr-1 levels after hormone and MAPK inhibitor treatment. The MEK1/2 inhibitor PD98059 completely blocked GnRH stimulation of Egr-1; in contrast, treatment with p38MAPK inhibitor SB202190 at a concentration that previously inhibited GnRH stimulation of the PP (Fig. 11C) had no effect on GnRH stimulation of Egr-1 levels (Fig. 12, panel B). We then did a pull-down assay using the two putative Egr-1 DNA binding sites encoded in the PP (Table 1) followed by Western blot analysis. Egr-1 bound the proximal (pEgr-1) -55/-32 and not the distal (dEgr-1) -114/-91 binding site (Fig. 12C). We could not detect any direct binding of Egr-1 to the dEgr-1 DNA binding site using this assay (data not shown), but binding of Egr-1 to the pEgr-1 DNA binding site was specific because binding was competed by wild-type pEgr-1 and not mutated mpEgr-1 double-stranded oligonucleotides (Fig. 12C). Pretreatment of L β T2 cells with MAPK inhibitors blocked Egr-1 DNA binding activity in the isolated nuclear extracts (Fig. 12D). This demonstrates that the PP contains one high-affinity pEgr-1 binding site and that DNA binding of Egr-1 is regulated by MAPK.

MAPK Regulates Egr-1 Activation of the LH β -Subunit Promoter

The regulatory role of Egr-1 transactivation of the PP was further investigated by point mutation of each DNA binding site to generate single and double distal and proximal mutated PP (mutated distal Egr-1, mdEgr-1; mutated proximal Egr-1, mpEgr-1; mutated distal proximal Egr-1, mdpEgr-1). These were stimulated in a series of transient transfection assays with GnRH and treated with a combination of either PD98059 or SB202190 or both (Fig. 13A). GnRH stimulated similar levels of expression of the PP and mdEgr-1, and inhibitor blockade indicated that promoter response was marginally impaired by mutation of the distal site. In contrast, GnRH failed to induce either the mpEgr-1 or mdpEgr-1 point-mutated construct; consequently, further addition of MAPK inhibitors had no significant effect. Thus, this indicated that the pEgr-1 DNA binding site dominantly regulated promoter activity. Cotransfection of a constitutively expressed Egr-1 induced high levels of PP basal gene expression, which was further augmented by addition of GnRH. But addition of either PD98059 or SB202190 or both abrogated this GnRH-induced stimulation of Egr-1 activity (Fig. 13B). Coexpression of Egr-1 with the md- and mpEgr-1 constructs did not induce high levels of basal promoter activity, but further addition of hormone did. The mdpEgr-1 promoter was refractory to both Egr-1 and the combination of Egr-1 and GnRH. Addition of the MEK1/2 and/or p38MAPK inhibitors blocked GnRH induction of Egr-1 transcriptional activity of both md- and mpEgr-1, but had no effect on mdpEgr-1. These data show that both ERK and p38MAPK augment Egr-1 transcriptional activity, and that Egr-1 transactivates the PP very weakly via a distal site and strongly via a proximal DNA binding site and is the main effector of GnRH-induced ERK-regulated transcriptional modulation of the PP.

DISCUSSION

In the present study we have shown that GnRHR activation results in the formation of a Pyk2-dependant multiprotein superstructure, which facilitates the activation of the ERK isoform of the MAPK family. This triggers nuclear translocation of active ERK that is decoded into transcriptional activation of the LH β -subunit gene promoter. Egr-1 was shown to be the ERK-regulatory target by modulation of transcriptional activity and DNA binding, mainly mediated via a proximal Egr-1 DNA binding site. Altogether, we have mapped a new ERK-signaling route from activation of GnRHR to activation of transcription.

We have shown that GnRH-induced ERK activation is rapid and prolonged in both α T3-1 and L β T2 cells and is proximal in a hierarchical signaling response that, in contrast to a recent report, does not occur through an EGFR-based mechanism (20). This discrepancy may be due to our use of a physiological 10 nM GnRH concentration to activate ERK; doses in excess of 200 nM will also activate ERK (20), but gonadotrope cells can desensitize the ERK-induced transcriptional apparatus if GnRH exceeds 100 nM (18,21). Hence, we observed no cross talk between EGFR and GnRH, because inhibition of the EGFR intrinsic kinase failed to attenuate GnRH-induced ERK activation, and there was negligible GnRH-induced tyrosine autophosphorylation of the EGFR. Thus, these data agree with others that EGFR does not contribute to the GnRH-induced ERK-signaling response in gonadotrope cells (5,19) and suggest that if EGFR cross talk is not the primary mode of MAPK activation (7), then the structural integrity of the cell cytoskeleton is key (9,11,44).

In this study GnRH-induced ERK activation appeared to contain cytoplasmic calcium and tyrosine kinase-dependent components. Although others have shown increased FAK activity in α T3-1 cells, this was not linked to ERK activation (5), and we noted only minimal activation of FAK in both α T3-1 and L β T2 cells; therefore FAK was excluded as a major ERK target in these cells. Because the FAK-related calcium-dependent tyrosine kinase Pyk2 is involved in ERK activation by many different types of GPCRs (7,12,45,46), we further investigated the role of this scaffolding protein in ERK activation. The tyrosine phosphorylation of Pyk2 and the sensitivity of the GnRH-induced ERK signal to disruptors of the actin cytoskeleton indicated that the ERK-activating signal was being transmitted through focal adhesion complexes. Chelation of intracellular calcium using BAPTA-AM perturbed Pyk2 activation and downstream ERK phosphorylation, supporting a role for Pyk2. The requirement for calcium in activation of ERK in gonadotropes is variable (13,47). This may reflect the different culture conditions used: we used low levels of serum and added GnRH, compared with serum-free conditions favored by others; this explains the difference in the level of ERK stimulation and possibly the reported degree of calcium dependence (13).

There was a clear relationship between ERK activation and tyrosine autophosphorylation of Pyk2: the two events were sensitive to the same panel of intracellular signaling pathway chemical inhibitors. In addition, GnRH induced the coassociation of Pyk2 with the downstream signaling factors, c-Src and Grb2. Grb2 is a bifunctional signaling adapter molecule possessing both Src homology-2 and -3 (SH2 and SH3) domains. The Ras-guanine nucleotide exchange factor GEF mSos associates exclusively with Grb2, and overexpression of dominant-negative forms of Pyk2 that were catalytically inactive or could not bind c-Src or Grb2 inhibited the ability of GnRH to activate ERK. Furthermore, we noted that a GnRH-stimulated focal adhesion-based mechanism was required to induce nuclear translocation of activated ERK. Taken together, these findings suggest that a multiprotein signaling complex assembles, comprising Pyk2, c-Src, and some components of the MAPK pathway, *i.e.* Grb2-Sos and ERK, and is dependent upon a functional actin cytoskeleton for selective translocation to the nucleus.

The nuclear locus of action for ERK was mapped and characterized as one of two GnRH-activated *cis*-acting DNA regions in the LH β -subunit promoter, the URR (−692 to −626 bp) and the PP (−136 to −29 bp). The URR was independently transcriptionally active from the PP and appeared to be regulated by JNK. The PP was important for regulation of an integrated GnRH response, because at this locus, inhibition of ERK and p38MAPK was additive, indicating convergence of distinct signal transduction pathways. ERK and p38MAPK could activate known or unknown transcription factors, and although the PP is activated by SF-1 and Pitx1 (35,36), levels do not alter in response to GnRH treatment (40,48), but they may be post-translationally modified (29,30).

Therefore, because the PP encodes two putative Egr-1 DNA binding sites (37), we speculated that Egr-1 was the main effector of GnRH-activated ERK- and p38MAPK-mediated transcriptional regulation. In α T3-1 cells Egr-1 is induced by GnRH treatment (39,40,49,50), the gene promoter of Egr-1 is regulated by ERK (41), and several coregulatory proteins facilitate Egr-1 activation of the LH β -subunit gene promoter (51,52). In L β T2 cells both Egr-1 gene expression and DNA binding were regulated by ERK and p38MAPK, and only the pEgr-1 site actually bound protein in a pull-down assay. Pull-down assays are specific, because proteins can adopt a native conformation when binding DNA and EMSA of the pEgr-1 and dEgr-1 DNA binding sites in multiple species determined that the binding affinity of Egr-1 is considerably higher for the proximal than the distal DNA binding site (53). Point mutation of the Egr-1 DNA binding sites within the PP showed that pEgr-1 was crucial for promoter activation after GnRH stimulation and after cotransfection of a constitutively expressed Egr-1 construct. Egr-1 transcriptional activity could be blockaded by ERK and p38MAPK inhibitors or by mutation of the pEgr-1. In contrast, the dEgr-1 DNA binding site accounted for a very small proportion of promoter activity and operated as an ancillary site. GnRH signaling induces Egr-1 gene expression and transcriptional activity in immature α T3-1 cells (28,41); in nongonadotrope cells Egr-1 is an immediate early gene regulated mainly by *de novo* induction of gene expression (54) but can be differentially phosphorylated or acetylated according to the stimulatory input (55-57). In mature L β T2 cells, we find that ERK and p38MAPK converge to regulate the DNA binding and transcriptional activity of Egr-1. Interestingly, cofactor p300 regulates Egr-1 signaling in prostate cells and is known to interact with Egr-1 in gonadotrope cells (52,57).

We have demonstrated that GPCR-mediated ERK activation by a focal adhesion-based mechanism controls the generation of a transcriptionally active, nonmitogenic pool of activated ERK (Fig. 14). In these gonadotrope cells, GnRH stimulates immediate assembly of an ERK-activating Pyk2 scaffold that requires a functional actin cytoskeleton to translocate it to the cell nucleus, where it exerts a transcriptional effect to differentially regulate the output of the LH β -subunit gene via Egr-1.

MATERIALS AND METHODS

Materials

The actin filament disruptors, cytochalasin D and latrunculin B, were obtained from Calbiochem (Merck Biosciences, Beeston, UK). GnRH, epidermal growth factor (EGF), and synthetic RGDS/RGES peptides and anti-Flag M2 agarose were obtained from (Peninsula Laboratories, St. Helens, UK; or Sigma, Dorset, UK). The c-Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*b*]pyrimidine (PP2) and the chemical inhibitors genistein, PD98059, SB203580, SB202190, Ro31-8220, BAPTA-AM, and wortmannin were all obtained from Calbiochem. The cDNAs encoding the mutant Pyk2 constructs were donated by Ivan Dikic. Dominant-negative Raf and Ras constructs were donated by Ulf Rapp, and dominant-negative MEK and JNK are described in Refs. 5, 58, and 59). The myc-tagged ERK2 construct was obtained from Eisuke Nishida, Kyoto University.

Generation of Constructs

The ovine LH β -subunit gene promoter 5'-flanking sequence -1000, -692, -626, -536, and -320-bp constructs were generated by Exonuclease III digestion of -1786-bp fragment (34) and were ligated upstream of a LacZ reporter in pNLacF (60). The pA3Luc luciferase reporter (a gift from Dr. David Gordon, Denver, CO) construct -692 was prepared using -1786 pNLacF as a template using Extensor Long PCR master mix (Abgene, Epsom, UK) and primer pair oS1/oAS1 (Table 1). Point-mutated constructs were generated by PCR using appropriate primer combinations mpEgr-1 (oS2/omAS2), mdEgr-1 (omS2/oAS2), and mdpEgr-1 (omS2/

omAS2) and incorporated into a larger fragment by additional denaturing, annealing, and priming cycles using an internal overlap with a 74-bp (oCS2/oACS2) fragment (Table 1). The resultant PCR products were cloned into pCRII vector (Invitrogen Ltd., Paisley, Scotland, UK) and excised with *KpnI/HindIII* or appropriate restriction enzymes and ligated into pA3Luc. Promoter constructs -320, -136, UrrΔPP1, URRΔPP2, and the minimal promoter (-29) were cloned into pA3LUC using defined restriction sites and standard methods. The Egr-1 protein expression construct was made as follows: the two individual exons of Egr-1 were amplified by PCR from 1 ng of human genomic DNA using exon-specific primers (Table 1). These PCR products were denatured and annealed using the introduced FLAG tag before amplification by PCR using the Egr-1ex1S and Egr-1ex2AS primers and cloned into pCDNA 3.1 (Invitrogen Ltd.). The reading frame and integrity of the Egr-1 construct were checked by *in vitro* translation using a TNT-coupled wheat germ extract (Promega UK, Ltd., Southampton, UK) according to manufacturer's instructions. All constructs were verified by DNA sequencing.

Cell Culture and Transfection

Murine α T3-1 and L β T2 gonadotrope cells were provided by Professor Pamela Mellon (San Diego, CA) and cultured as described elsewhere (32). To serum deprive gonadotropes, cell monolayers were incubated with DMEM supplemented with 0.5% fetal bovine serum for 16 h. Transient transfections were either performed using Superfect (Qiagen House, Crawley, UK) as described previously (61) or were carried out as described in Refs. 21 and 36. LacZ transfections used chloramphenicol acetyl transferase (CAT) plasmid pCMV-CAT as an internal control for transfection efficiency. Human Egr-1/pCDNA3.1 expression vector was cotransfected at a ratio of 1:200; dnMEK was cotransfected at a ratio of 1:10. After 48 h the cells were treated for a further 6 h with 100 nM GnRH or were left untreated. Chemical inhibitors were added for the time periods specified in the figure legends before agonist stimulation. CAT activity was determined as described elsewhere (34), and LacZ activity was measured using a kit (Roche Diagnostics). Reporter activity was measured as described previously (21,36). The total reporter gene activity normalized against internal reference activity was taken as 1, and results were calculated as fold activation by GnRH over untreated levels. Data are presented as the mean \pm SEM. Statistical significance was determined using one-way ANOVA, with a value of $P < 0.05$ considered significant.

Measurement of MAPK Phosphorylation

Specific ERK1/2 or myc-ERK2 phosphorylation was measured as previously described (8,9). Visualization of alkaline phosphatase-labeled proteins was performed using enzyme-linked chemifluorescence (Amersham Pharmacia Biotech, Little Chalfont, UK) and quantified using a Storm 860 Phosphorimager. In Fig. 2 L β T2 cells were stimulated with GnRH and then treated essentially as described elsewhere (18), except 30- μ g sample aliquots were separated on 10% SDS-PAGE, followed by Western blotting with mouse monoclonal anti-phospho MAPKs (5, 16). Total MAPKs were detected with polyclonal antibodies for the various MAPKs as a control for sample loading.

Immunoprecipitation, Immunoblotting, and Egr-1 Pull-Down Assay

After stimulation cell monolayers were placed on ice, washed twice in ice-cold Dulbecco's PBS (DPBS), nuclear proteins were harvested as described (36), and a 25- μ g aliquot of this was fractionated by SDS-PAGE. Alternatively, if total protein extracts were required, the cells were washed and then lysed in an Nonidet P-40-based solubilization buffer (8), clarified by centrifugation at $10,000 \times g$ for 15 min, and diluted to 1 mg/ml of total protein. A 50- μ l aliquot of clarified lysate was mixed with an equal volume of 2 \times Laemmli sample buffer and resolved by SDS-PAGE for confirmation of plasmid expression or determination of intracellular protein activation by protein immunoblotting.

Phosphotyrosine proteins were immunoprecipitated with 20 μ l of a 30% slurry of anti-PY20 agarose preconjugated antisera (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with agitation for 16 h at 4 C. Pyk2 was immunoprecipitated using 1 μ g of mouse antihuman Pyk2 monoclonal antisera (BD Biosciences, Oxford, UK) with 25 μ l of a 50% slurry of protein G-plus/protein A agarose with agitation for 16 h at 4 C. Grb2 was immunoprecipitated with 2 μ g of rabbit antihuman Grb2 polyclonal antisera (Santa Cruz), and 25 μ l of the protein G-plus/protein A agarose with agitation for 16 h at 4 C. Myc-epitope-tagged proteins were immunoprecipitated with 25 μ l of a 30% slurry of anti-myc agarose preconjugated antisera (Santa Cruz) with agitation for 16 h at 4 C. Immune complexes were detected as described previously (9).

Endogenous epidermal growth factor receptor (EGFR) was detected using a 1:1000 dilution of a rabbit antimouse EGFR polyclonal IgG (Santa Cruz), Grb2 was detected with a 1:1000 dilution of a rabbit antihuman Grb2 polyclonal IgG (Santa Cruz), and Egr-1 was detected by a 1:5000 dilution of a rabbit antihuman polyclonal IgG (Santa Cruz). Both EGFR and Grb2 used a 1:15,000 dilution of alkaline phosphatase-conjugated polyclonal antirabbit as a secondary antibody (Sigma). For Egr-1 a 1:20,000 dilution of horseradish peroxidase-conjugated swine antirabbit IgG was used, and proteins were visualized as described (62). Tyrosine-phosphoproteins were detected using a 1:1000 dilution of a mouse antimouse PY99 (Santa Cruz) and a 1:15,000 dilution of alkaline phosphatase-conjugated polyclonal antimouse as a secondary antibody (Sigma).

For Egr-1 pull-down assays, 250 μ g of M280 streptavidin Dynabeads (DynaL Biotech Ltd., Wirral, UK) were mixed with 20 pmol of biotinylated distal (d) or proximal (p) Egr-1 double-stranded DNA oligonucleotides (Table 1) at room temperature for 1 h. Unbound DNA was washed off before addition of 150 μ g of L β T2 nuclear extract in binding buffer 1 (20 mM Tris-HCl, pH 7.9; 50 mM NaCl; 10% glycerol; 1 mM EDTA, pH 8.0; 1 mM dithiothreitol) with Complete protease inhibitors (Roche Diagnostics). Where appropriate, a 25-fold excess of nonbiotinylated competitor double-stranded oligonucleotide was preincubated with the nuclear extract. The binding reactions were incubated with constant rocking at 4 C for 4 h, after which excess protein was washed off, and bound complexes were eluted in 10 μ l of 0.25M NaCl and 0.0125% SDS, resolved by SDS-PAGE, and detected by Western blotting.

Immunocytochemistry and Confocal Microscopy

Confocal microscopy was performed using a Zeiss LSM510 laser-scanning microscope using a 63×1.4 numerical aperture oil immersion lens. L β T2 cells were stimulated for the time periods specified in the appropriate figure legends with GnRH (10 nM) at 37 C. After stimulation, the cells were washed in DPBS, fixed in 100% methanol (MeOH) at -20 C for 10 min, permeabilized at room temperature using DPBS supplemented with 1% BSA and 0.2% NP-40 for 30 min and eventually mounted in Permafluor (Immunotech). The permeabilized cells were then blocked for 1 h with a 1% BSA-DPBS solution. After blocking, the permeabilized cells were then incubated for 1 h with a 1:200 dilution of a rabbit antiactive ERK1/2 followed by an antirabbit fluorescein isothiocyanate-conjugated secondary (1:100 dilution).

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Abbreviations

BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester; CAT, chloramphenicol acetyl transferase; dnMEK, dominant-negative MEK; DPBS, Dulbecco's PBS; EGFR, epidermal growth factor receptor; Egr-1, early growth response-1; FAK, focal adhesion kinase; GnRHR, GnRH receptor; GPCR, G protein-coupled receptor; JNK, c-Jun NH₂-terminal kinase; mdEgr-1, mutated distal Egr-1; mdpEgr-1, mutated distal proximal Egr-1; mpEgr-1, mutated proximal Egr-1; MEK, MAPK kinase; PP, proximal promoter; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-b]pyrimidine; Pyk2, proline-rich tyrosine kinase 2; RGDS, Arg-Gly-Asp-Ser; RGS, Arg-Gly-Glu-Ser; SF-1, steroidogenic factor-1; URR, upstream response region.

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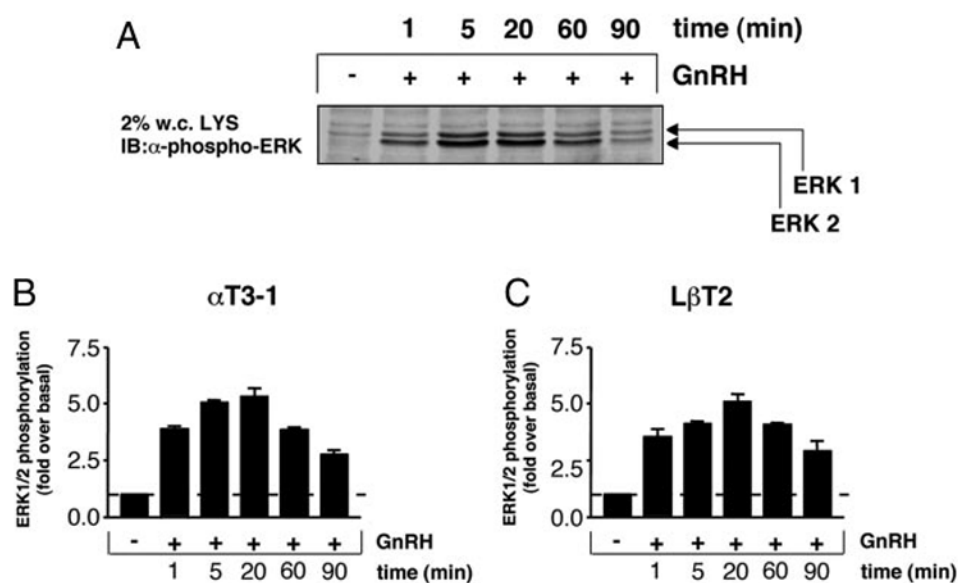


Fig 1. Sustained ERK Activation in Gonadotrope Cell Lines

GnRH stimulation of α T3-1 or L β T2 cells results in a rapid elevation of the cellular amount of the active form of the ERK1/2 MAPK isoform. A, Representative antiactive ERK1/2 Western blot of 2% of whole-cell extract from L β T2 cells. B and C, Histograms of mean \pm SEM data from at least four individual measurements of GnRH-induced ERK1/2 activation in α T3-1 and L β T2 cells. IB, Immunoblot; W.C. LYS, whole-cell lysate.

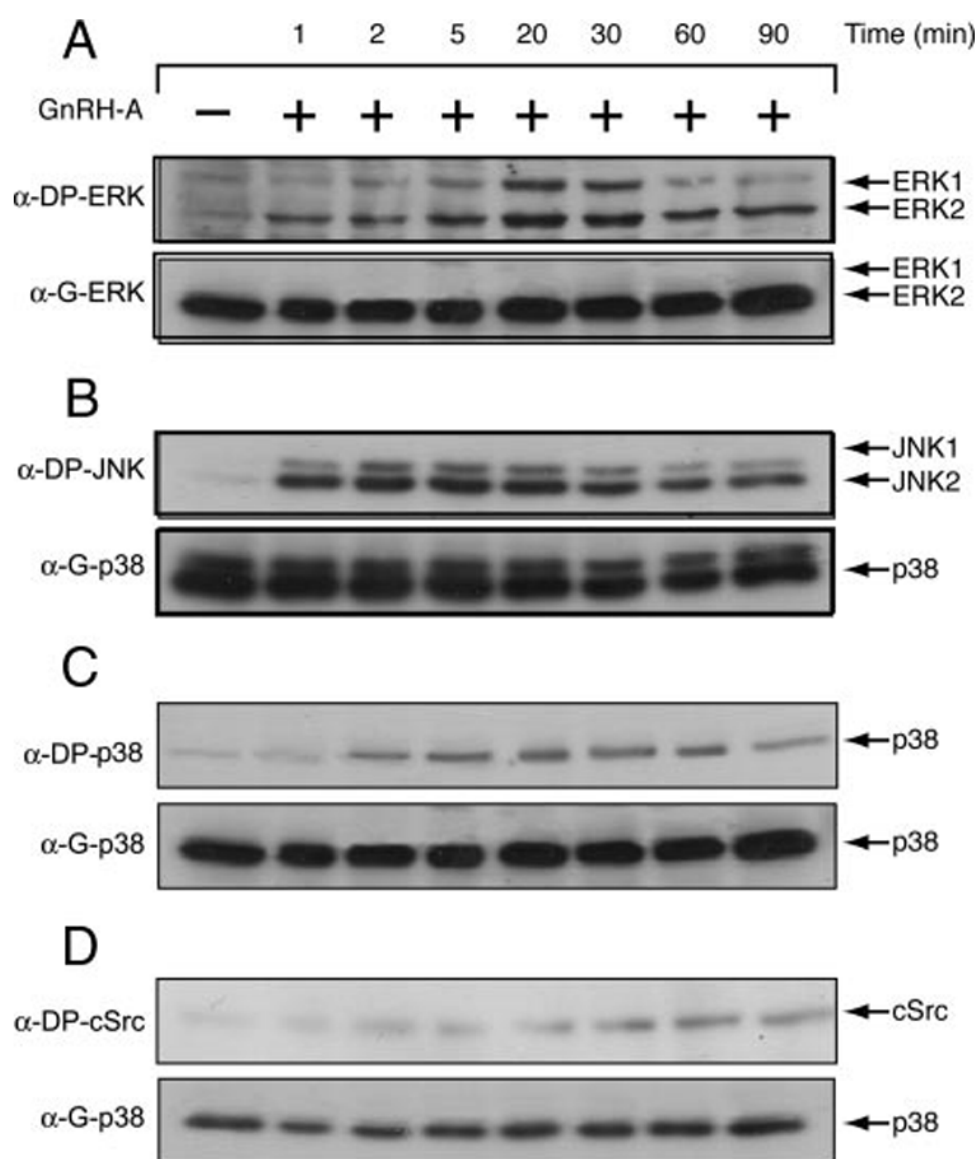


Fig 2. MAPK Activation in $L\beta T2$ Gonadotrope Cell Line

GnRH stimulation of $L\beta T2$ cells activates MAPK signaling in the form of ERK1/2 and JNK1/2 isoforms and p38MAPK and c-Src. A, Representative antiactive ERK1/2. α -DP, Anti-diphospho; α -G, anti-general. B, Rapid elevation of antiactive JNK1/2 for comparison. C, Induction and time course of activation of active p38MAPK. D, Activation of c-Src over the same time frame as panels A–C. In each experiment a Western blot of 2% of whole-cell extract from $L\beta T2$ cells was used. Panels B–D used p38MAPK as a loading control, whereas panel A used the nonactive form of ERK1/2. The panels shown are representative of three separate experiments.

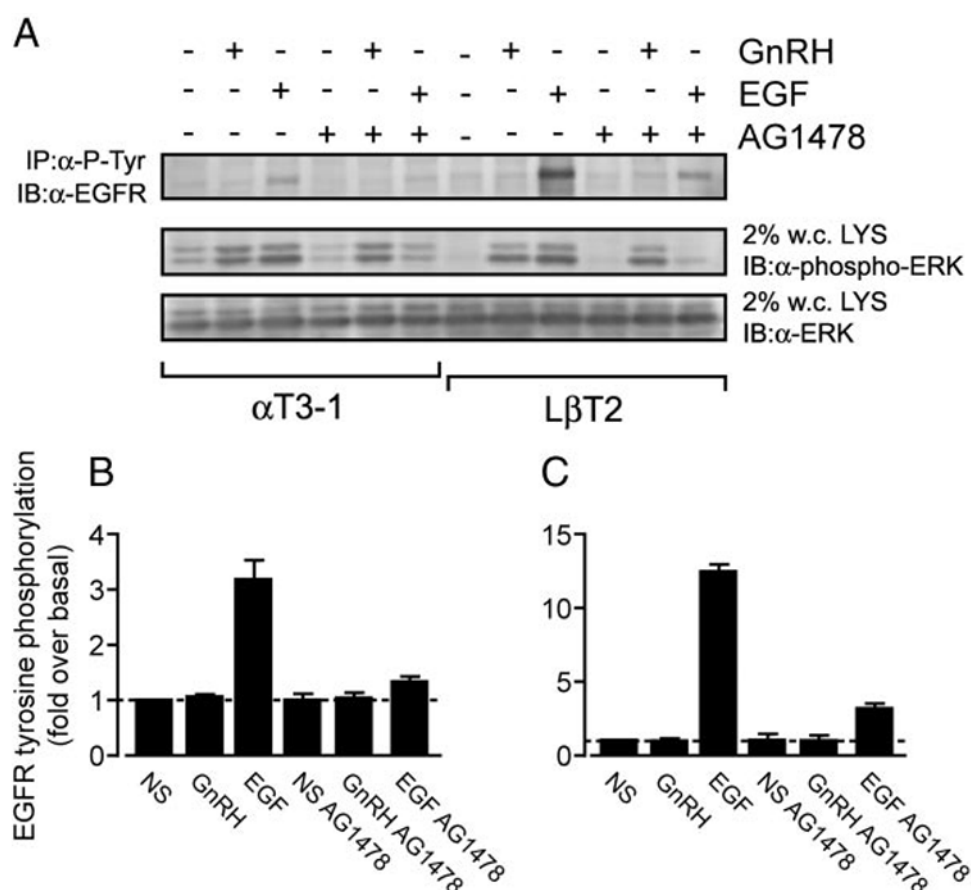


Fig 3. GnRHR Activation Mediates ERK1/2 Activation Independently of EGFR Kinase Activity
 A, EGF (10 ng/ml, 10 min) but not GnRH (10 nM, 10 min) elevated the tyrosine phosphorylation status of the immunoprecipitated EGFR in both α T3-1 and L β T2 cells. Preincubation of α T3-1 and L β T2 cells with tyrphostin AG1478 inhibited EGF (10 ng/ml, 10 min)-induced EGFR tyrosine phosphorylation. AG1478 pretreatment effectively inhibited EGF-induced but not GnRH-induced ERK1/2 activation. The effects of GnRH and EGF upon EGFR tyrosine phosphorylation in α T3-1 and L β T2 cells are quantified in panels B and C, respectively. Each bar in the histograms in panels B and C depict mean \pm SEM data values for the degree of both ERK1/2 activation and EGFR tyrosine phosphorylation by EGF and GnRH in the presence or absence of AG1478 from three separate experiments. IB, Immunoblot; IP, immunoprecipitation; NS, nonstimulated; w.c. LYS, whole-cell lysate.

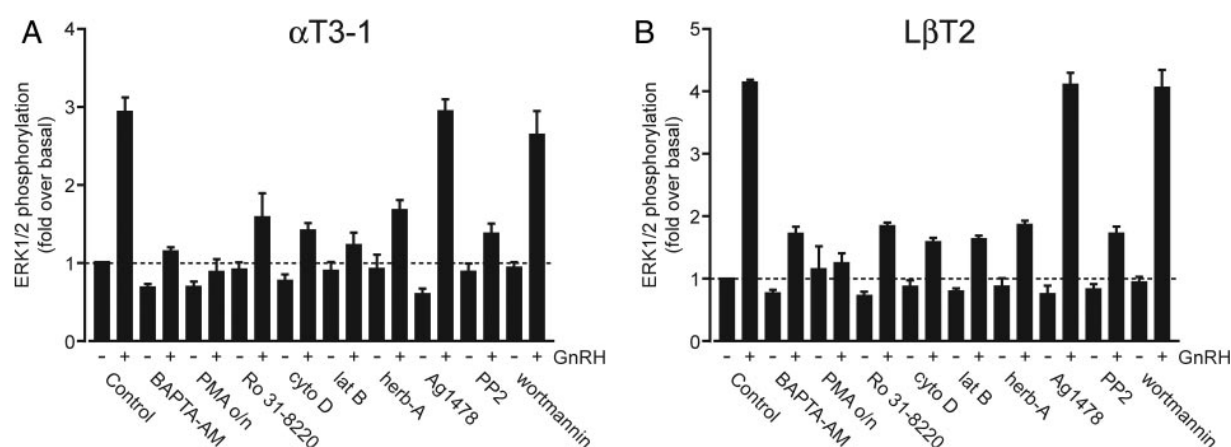


Fig 4. Chemical Sensitivity of ERK1/2 Activation in Gonadotrope Cells

Both α T3-1 (panel A) and L β T2 cells (panel B) were preincubated with BAPTA-AM (50 μ M, 30 min), phorbol 12-myristate-13-acetate (1 μ M, 16 h), Ro-31 8220 (100 nM, 60 min), cytochalasin D (1 μ M, 60 min), latrunculin B (1 μ M, 60 min), herbimycin-A (1 μ M, 60 min), tyrphostin AG1478 (100 nM, 30 min), PP2 (1 μ M, 30 min), and wortmannin (10 nM, 30 min) before a 10-min stimulation with 10 nM GnRH. A similar general pattern of chemical inhibitor sensitivity was observed between α T3-1 and L β T2 cells. Hence, on both cell types the GnRH-induced ERK1/2 activation appeared to be dependent upon intracellular Ca^{2+} elevation, PKC activity, intact cytoskeletal structure, and c-Src tyrosine kinase activity yet relatively insensitive to inhibition of EGFR tyrosine kinase activity or phosphatidylinositol 3-kinase activity. In both panels A and B, each bar in each histogram represents the mean \pm SEM of three individual experiments.

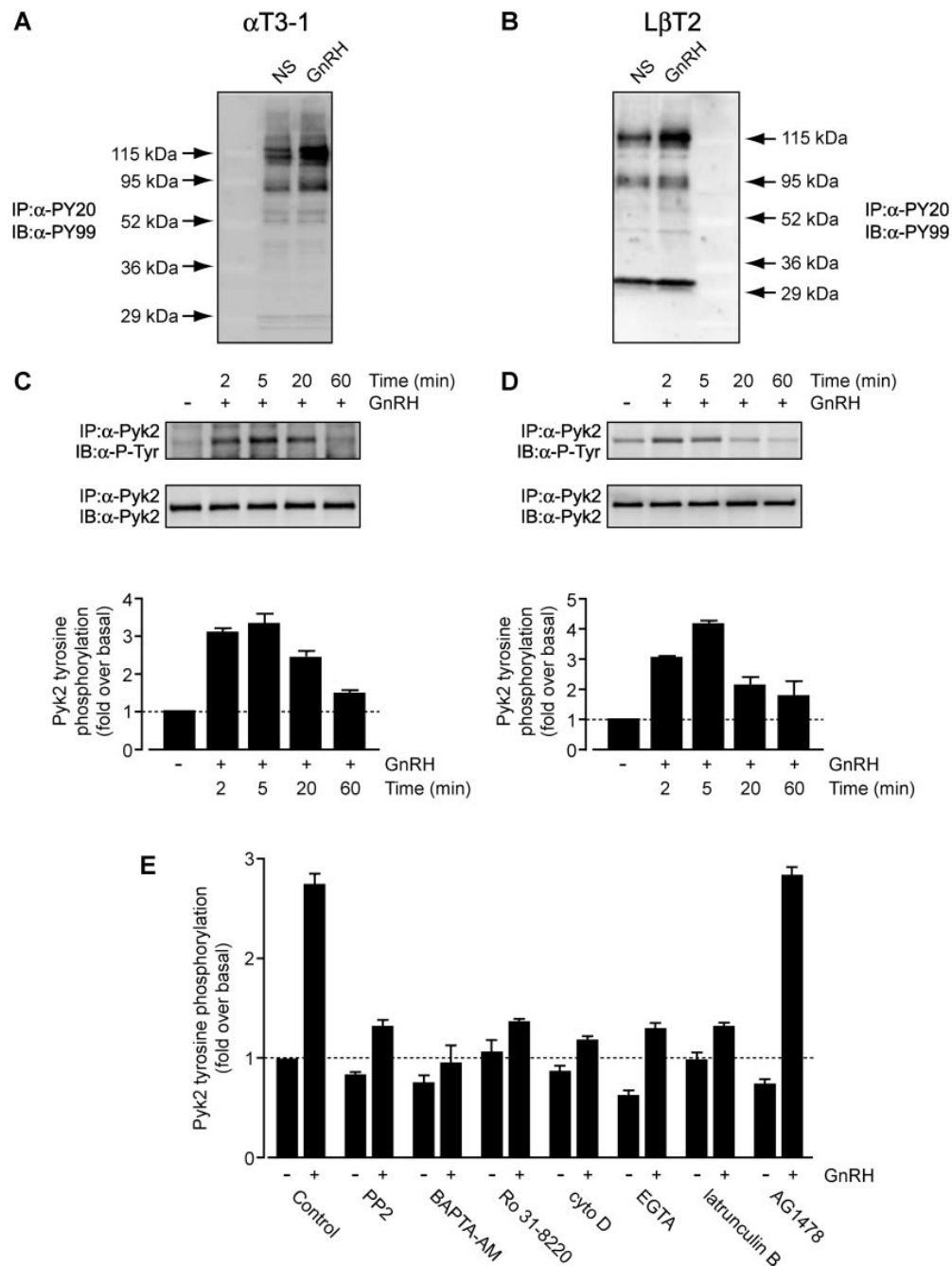


Fig 5. GnRH Activation Induces the Activation of the FAK Pyk2 in Gonadotrope Cells
 A and B, Representative Western blots of antiphosphotyrosine immunoprecipitates (PY20) probed with an antisera also against phosphotyrosine residues (PY99). In both panels several proteins can be identified increasing in their phosphotyrosine content in response to GnRH, but the most profoundly affected protein migrates with a mass close to the β -galactosidase 115-kDa molecular mass marker. GnRH stimulation resulted in a time-dependent increase in the degree of tyrosine phosphorylation of directly immunoprecipitated Pyk2 (panel C, α T3-1; panel D, L β T2). The bars in each histogram in panels C and D represent the mean \pm SEM of three individual Pyk2 immunoprecipitation experiments. GnRH-mediated elevation in Pyk2 tyrosine kinase activity was efficiently inhibited by PP2 (1 μ M, 30 min), BAPTA-AM (50 μ M, 30 min),

Ro-31 8220 (100 nM, 60 min), and cytochalasin D (1 μ M, 60 min), but not by tyrphostin AG1478 (100 nM, 30 min) pretreatment. Pyk2 activation demonstrates a similar chemical sensitivity to the ERK1/2 activation pathway and occurs largely independently of EGFR activity. Each *bar* in the histogram represents the mean \pm SEM of at least three individual experiments. IB, Immunoblot; IP, immunoprecipitation; NS, nonstimulated.

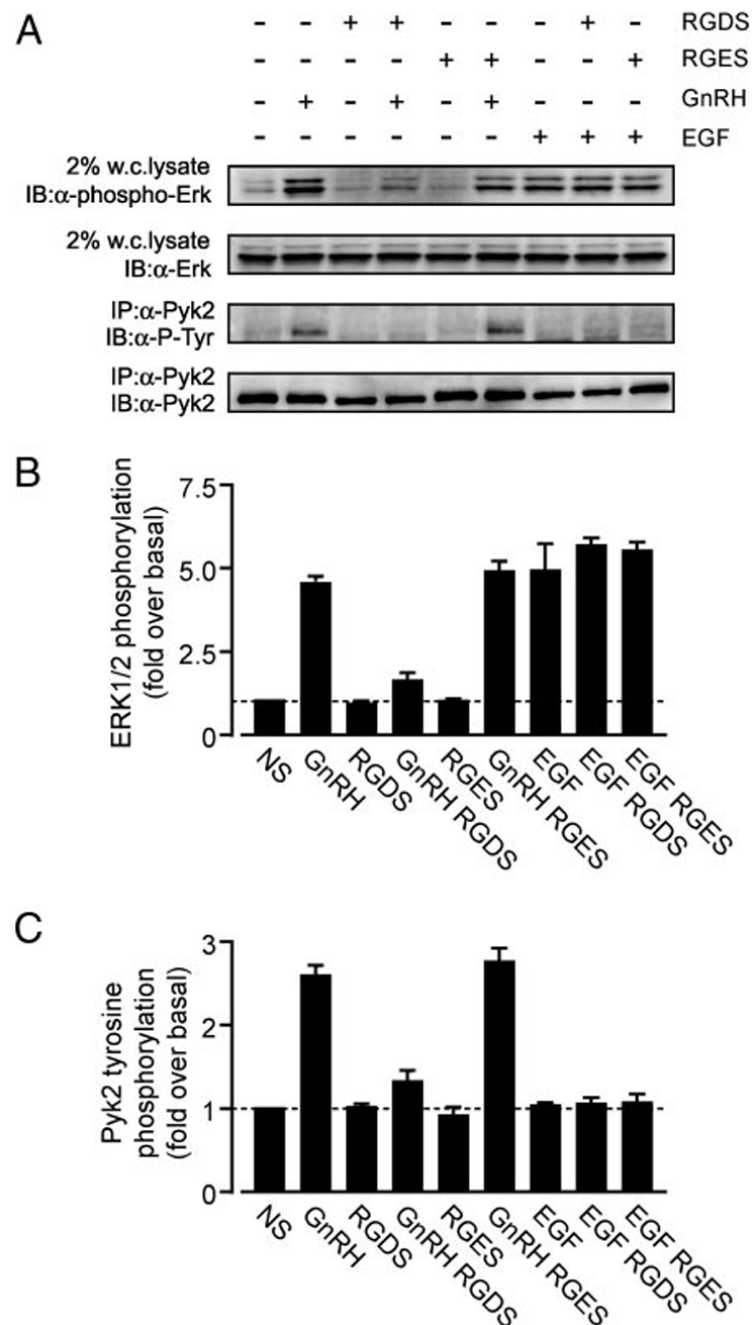


Fig 6. Focal Adhesion Dependence of GnRH-Induced Pyk2 and ERK1/2 Activation

LβT2 cells were pretreated with either 1 mM RGDS or 1 mM RGES for 16 h before EGF (10 ng/ml, 10 min) or GnRH stimulation (10 nM, 10 min). A, RGDS abrogated GnRH-induced, but not EGF-induced, ERK1/2 and activation. Preincubation with the control RGES tetrapeptide had no effect on either GnRH- or EGF-induced ERK1/2 activation. RGDS pretreatment attenuated GnRH-induced Pyk2 tyrosine phosphorylation, whereas RGES had no effect. EGF stimulation failed to alter the tyrosine phosphorylation status of immunoprecipitated Pyk2. The histograms in panel B and C depict mean \pm SEM. ERK and Pyk2 phosphorylation data are from at least three separate experiments. IB, Immunoblot; IP, immunoprecipitation; w.c., whole cell; NS, nonstimulated.

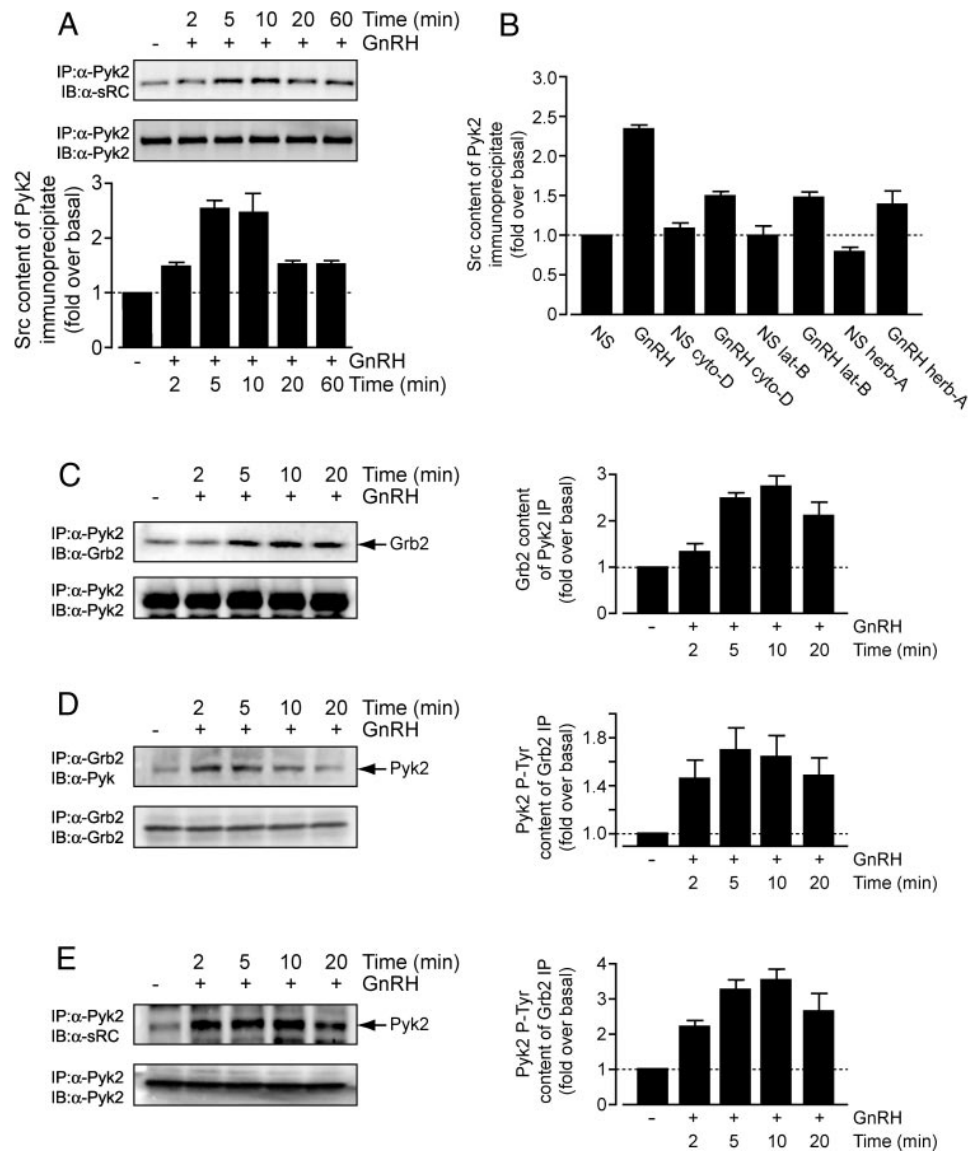


Fig 7. GnRH-Induced Association of Pyk2 with Downstream Signaling Intermediates

A, Representative Western blot for the c-Src content of Pyk2 immunoprecipitates from $L\beta T2$ cells. With GnRH stimulation there is a time-dependent association between c-Src and Pyk2. B, GnRH-induced c-Src and Pyk2 association is inhibited by cytochalasin-D, latrunculin-B, and herbimycin-A. GnRH induces a dynamic elevation of the relative Grb2 content of Pyk2 immunoprecipitates (panel C). GnRH stimulation also induces a dynamic elevation of the relative Pyk2 content of Grb2 immunoprecipitates (panel D). GnRH stimulation induces a dynamic elevation of the relative tyrosine-phosphorylated-Pyk2 content of Grb2 immunoprecipitates (panel E). The associated histograms display mean \pm SEM data of at least three individual experiments. cyto-D, Cytochalasin-D; herb-A, herbimycin-A; IB, immunoblot; IP, immunoprecipitation; lat-B, latrunculin-B; NS, nonstimulated.

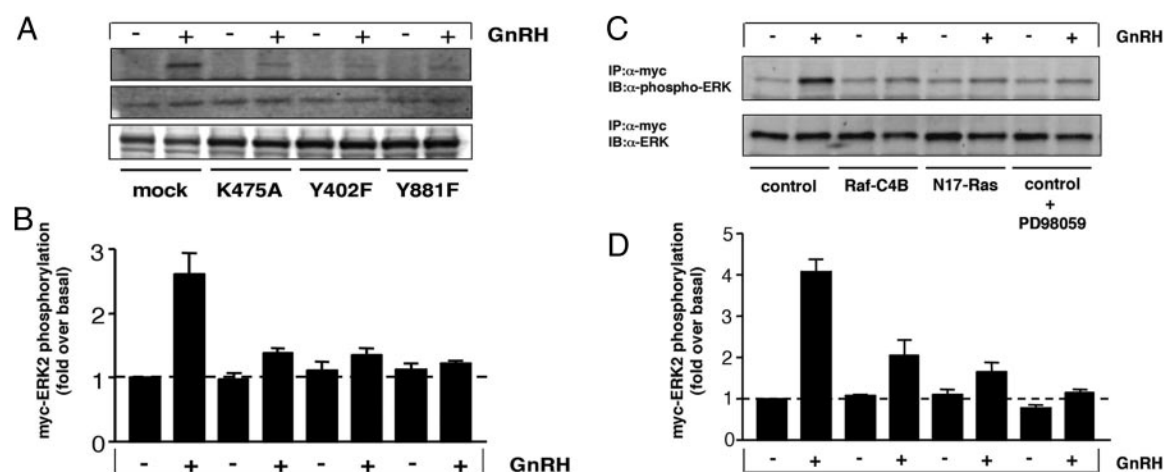


Fig 8. Activation of ERK1/2 Requires Protein Complex Assembly at the Pyk2 Tyrosine Kinase and Occurs via a Canonical Ras-Dependent Cascade

GnRH stimulation (10 nM, 10 min) elevated the degree of active transiently transfected myc-tagged ERK2 (panel A). Cotransfection with a kinase-deficient (K475A), a c-Src binding-deficient (Y402F), and a Grb2 binding-deficient form of Pyk2 (Y881F) inhibited GnRH-mediated activation of the myc-ERK reporter. The associated histograms (panel B) represent the mean \pm SEM of myc-ERK2 activations gathered from four individual experiments. In panel C, GnRH-induced (10 nM, 10 min) activation of myc-ERK2 was also abrogated when dominant-negative Ras (N17Ras) or Raf (Raf-C4B) were coexpressed. Pretreatment with the chemical MEK1/2 inhibitor PD98059 (20 μ M, 60 min) before GnRH stimulation also significantly reduced the GnRH-induced myc-ERK2 activation. The histogram in panel D depicts mean \pm SEM ERK2 activation data gathered from four individual experiments performed as in the Western blot (panel C). IB, Immunoblot; IP, immunoprecipitation.

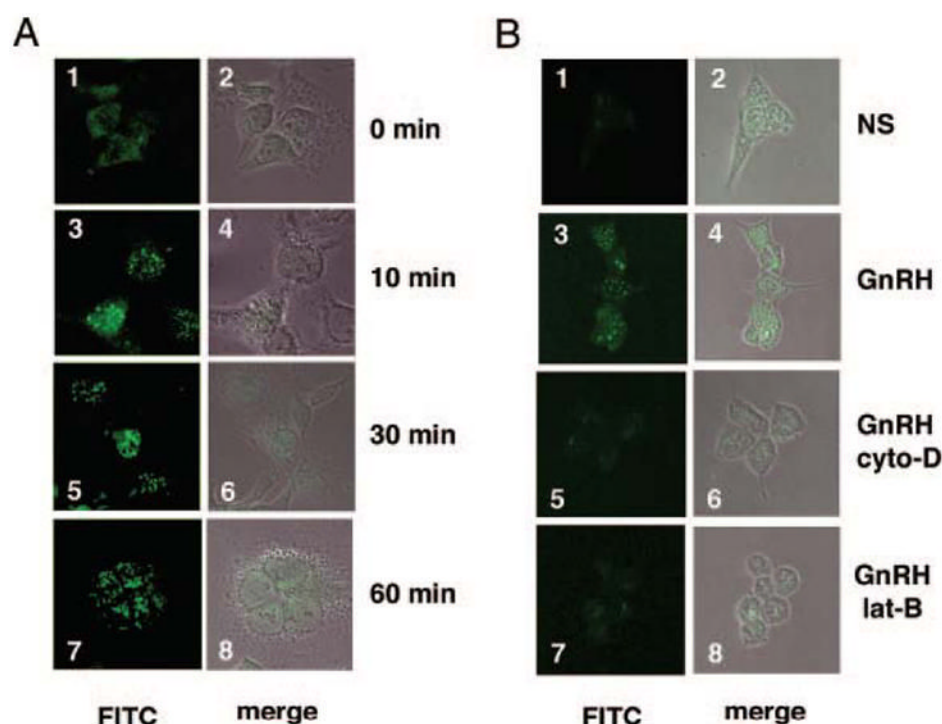


Fig 9. GnRH Mediates the Time-Dependent Activation of ERK1/2 in the Gonadotrope Nucleus
 GnRH stimulation of LβT2 cells (10 nM, ≤90 min) resulted in the generation of an increase in the cellular antiactive ERK1/2 immunoreactivity. The images in panel A depict representative confocal laser images from permeabilized LβT2 cells stained for antiactive ERK1/2. In the absence of ligand (images 1–2) there is minimal activated ERK1/2 staining; however, with 10 min of 10 nM GnRH stimulation there appears to be an increase in the cytoplasmic active ERK1/2 staining and the generation of an annular nuclear staining pattern (images 3–8). In panel B (images 5–8), the degree of nuclear active ERK1/2 is significantly attenuated when the cells were pretreated with cytochalasin-D (1 μM) or latrunculin-B (1 μM). cyto-C, Cytochalasin-D; lat-B, latrunculin-B; FITC, fluorescein isothiocyanate.

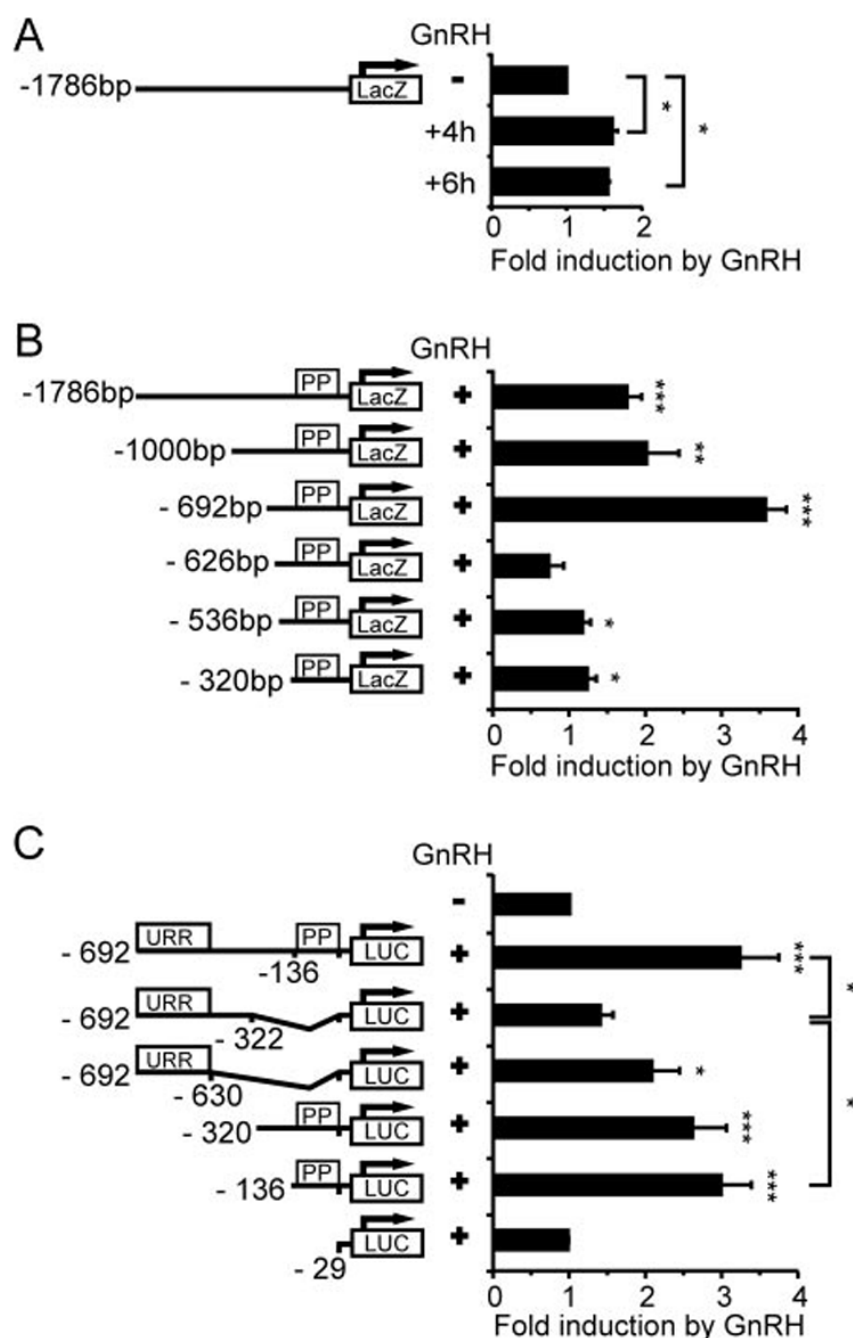


Fig 10. Mapping GnRH-Responsive Regions in the LH β -Subunit Promoter

A, 6 h of GnRH (100 nM) treatment of L β T2 cells induced the optimal GnRH response in a transfected -1786 ovine LH β -subunit gene promoter fragment ligated to a β -galactosidase (LacZ) reporter vector. Results are presented as fold induction of promoter activity by GnRH, calculated as LacZ/CAT (internal control) levels in untreated cells (-), expressed as 1, compared with the same construct in treated cells (+). B, GnRH treatment of a series of 5'-promoter resections identified a distal URR between -692 and -626 bp and a proximal region (PP) bounded by -320 bp. Results were calculated as described above. C, Internal deletion analysis of the -692-bp LH promoter ligated to a luciferase (LUC) reporter identified that the URR was independently responsive to GnRH. Hence, the URR and PP were mapped to -692/

–626 and –136 bp, respectively, and a negative regulatory *cis*-acting element was identified between –630 and –322 bp. Each panel is the average of at least three experiments, each done in triplicate. Significant differences were calculated using one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ untreated vs. treated, except where indicated. Results are expressed as the mean \pm SEM.

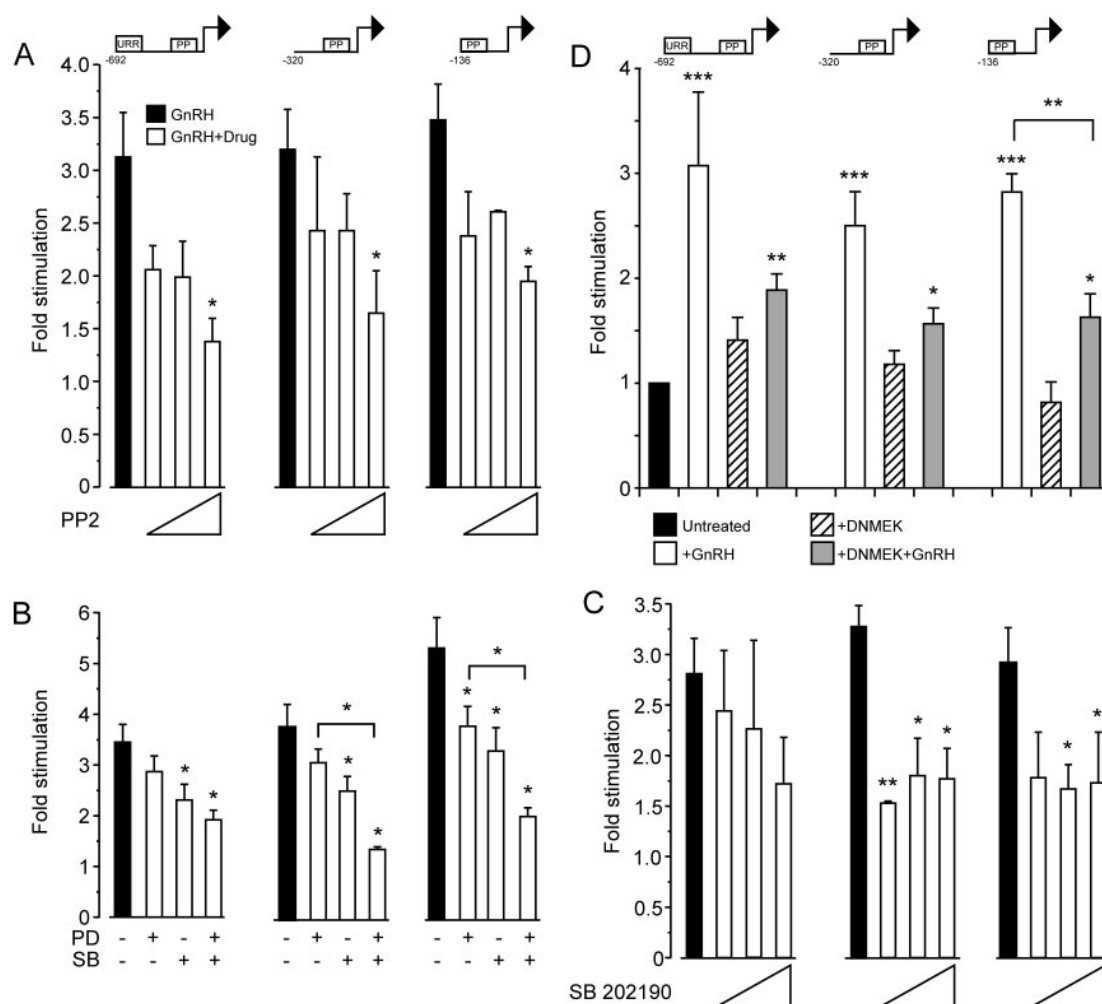


Fig 11. Differential MAPK Signaling to the LH β -Subunit Gene Promoter

A–C, LH β -subunit promoter constructs corresponding to –692 (URR and PP), –320 (PP), and –136 (PP) were transfected into L β T2 cells, pretreated with inhibitor where indicated, and either left untreated or treated with GnRH and incubated for a further 6 h. Luciferase levels were corrected for transfection efficiency and adjusted to 1 for untreated controls. *Black bars* correspond to control GnRH-treated, whereas *white bars* depict GnRH+drug. D, Constructs detailed above were cotransfected with 1:10 dnMEK expression plasmid; *white and gray bars* are treated with GnRH; *striped and gray bars* were cotransfected. One-way ANOVA identified the following: ***, $P < 0.001$; **, $P < 0.01$; and *, $P < 0.05$ as statistically significant from GnRH treated in panels A–D. GnRH fold stimulation is shown for each construct and was calculated from at least three separate experiments, each done in triplicate and expressed as mean \pm SEM. Statistical differences within treatment groups are also indicated where relevant. A, GnRH transactivation of the LH β -subunit promoter is uniformly and significantly inhibited by the tyrosine kinase inhibitor PP2 at 10- μ M concentrations. Inhibitor PP2 was added 30 min before GnRH in increasing concentrations (1, 5, and 10 μ M). B, 30-min pretreatment with MEK1/2 inhibitor PD (PD98059, 20 μ M) confirmed the sensitivity of the PP and indicated that inhibition of p38MAPK/JNK by SB (SB203580, 30 μ M) was additive at this site. In panel C the response to increasing concentrations of JNK/p38MAPK inhibitor SB202190 (1, 5, and 10 μ M) showed that the –692 promoter encoding the intact URR was less sensitive to JNK/

p38MAPK inhibition than the PP. D, Cotransfection with dnMEK mapped the ERK response to the PP.

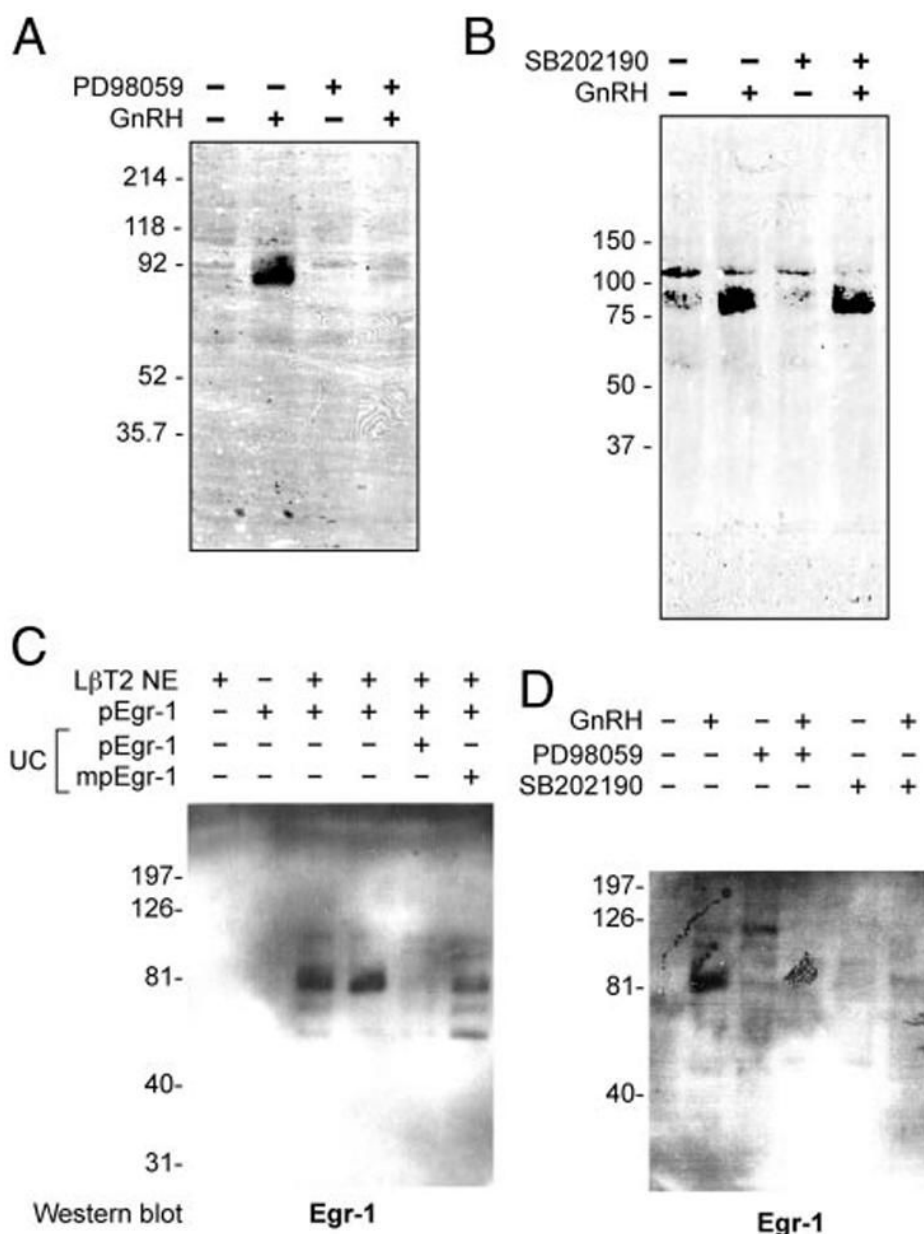


Fig 12. Egr-1 is the Transcription Factor Target of ERK-Mediated Signaling

LβT2 cells were left untreated (-) or treated with GnRH and/or either PD98059 or SB202190 (+) and nuclear proteins harvested 2 h later. A and B, Nuclear protein (25 µg) was size fractionated and Egr-1 expression detected with anti-Egr-1 antisera. Treatment of LβT2 cells with 50 µM specific MEK1/2 inhibitor PD98059, but not 5 µM p38MAPK inhibitor SB202190, blocks GnRH activation of Egr-1 protein expression. C and D, Nuclear extract (150 µg) was incubated with streptavidin-immobilized double-stranded biotinylated oligonucleotides encoding the proximal Egr-1 (pEgr-1) binding site for 4 h at 4 °C. Unbound competitor (UC) wild-type and mutant (m) double-stranded oligonucleotides were incubated with nuclear proteins at 25-fold excess. Complexes were then eluted and resolved by SDS-PAGE before transferring to PVDF and probing with antisera to Egr-1. C, The pEgr-1 sequence specifically bound Egr-1, which could be competed by wild-type but not mpEgr-1. D, The pEgr-1 DNA binding site was incubated with nuclear extract (NE) that had been pretreated with 50 µM

PD98059 or 5 μM SB202190 before addition of 100 nM GnRH. Inhibitor blockade also blocked Egr-1 binding to pEgr-1. Each panel is representative of an experiment that was repeated two times. Protein size markers are indicated in kilodaltons.

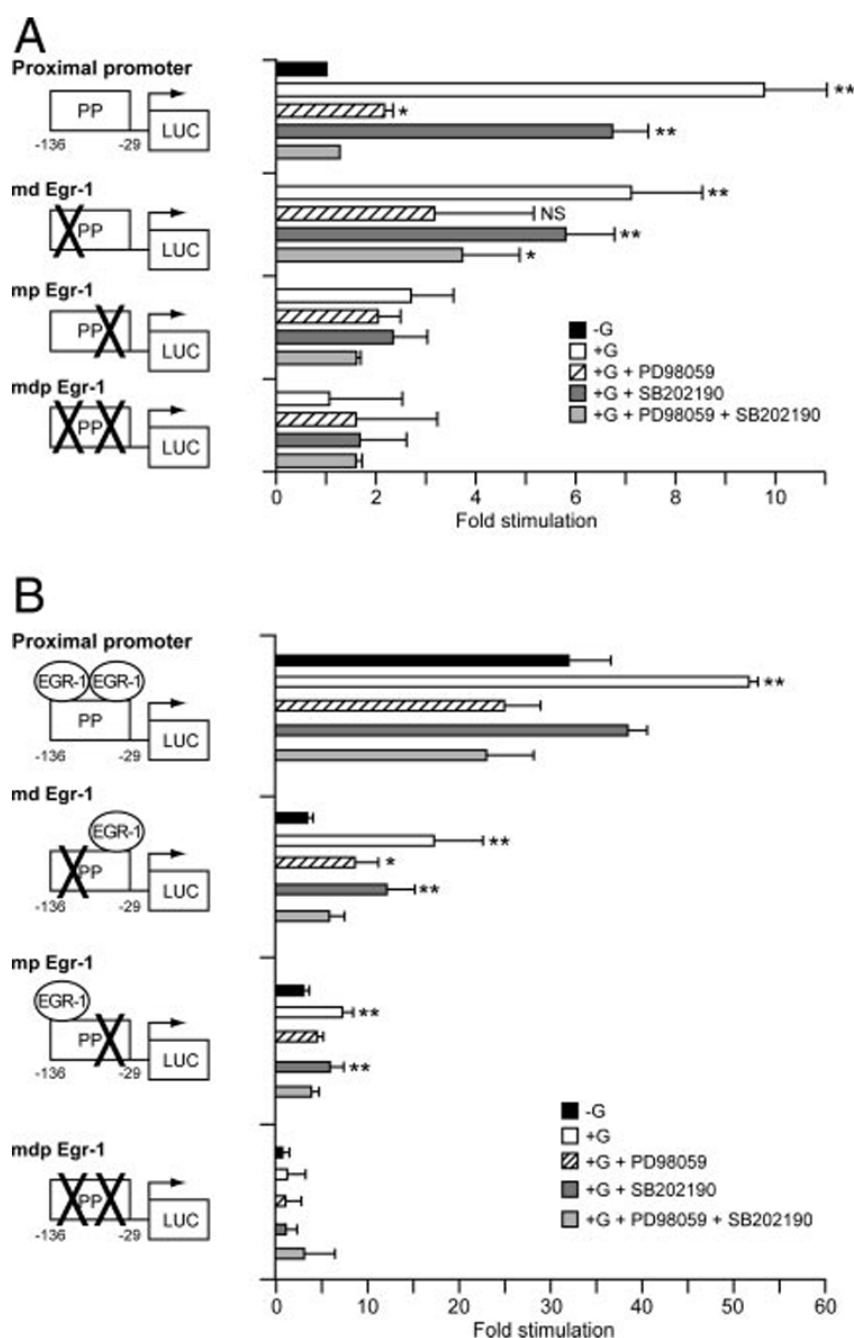


Fig 13. Egr-1 Specifically Transactivates the PP

L β T2 cells were transfected with either an intact PP or one point mutated at one of two possible Egr-1 DNA binding sites (indicated by a *black cross*), (mdEgr-1, mpEgr-1, and mdpEgr-1) luciferase reporter vectors (panel A), and cotransfected with a constitutive Egr-1 expression construct (panel B). Cells were then either left untreated or pretreated with MAPK inhibitors (PD98059 50 μ M, SB202190 5 μ M, or both) as indicated before being stimulated with 100 nM GnRH. A, GnRH induced a significant increase in luciferase levels that could be blocked by pretreatment with MAPK inhibitors. Mutation of mpEgr-1 or mdpEgr-1, but not mdEgr-1, blocked GnRH activation and rendered the inhibitors ineffective. B, Cotransfection of a constitutively expressed Egr-1 expression construct (1:200) induced basal PP expression,

which was augmented by GnRH. Pretreatment with MAPK inhibitors blocked GnRH-induced transcriptional activation of Egr-1 but had no effect on Egr-1 stimulation of basal promoter expression. Point mutation of either Egr-1 DNA binding site abrogated Egr-1-induced basal promoter activity; however, addition of GnRH activated the mdEgr-1, partially rescued mpEgr-1, and had no effect on mdpEgr-1. Consequently, MAPK blockade also mirrored this hierarchy. Each panel is the average of three separate experiments each done in triplicate. Significant differences within and between groups were determined by one-way ANOVA as *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$; and nonsignificant (NS) are indicated.

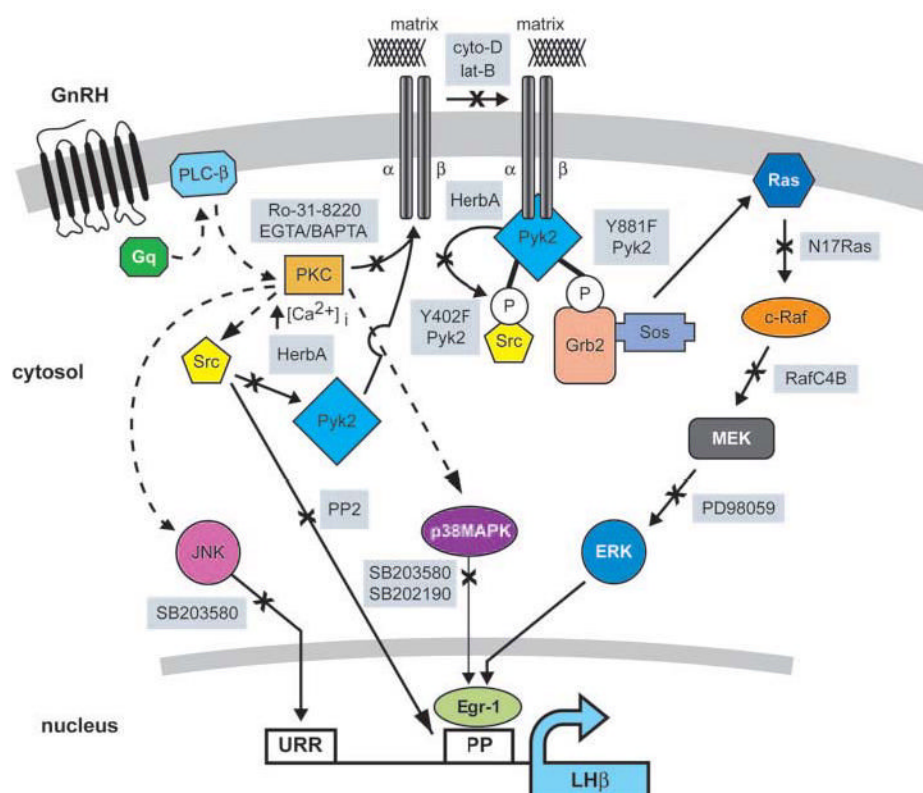


Fig 14. Schematic Representation of GnRH-Induced Pyk2-Dependent ERK-Activated Signaling to the LH β -Subunit Gene Promoter

Binding of GnRH to the GnRH-R triggers an intracellular signaling cascade, which radiates from activation of PKC. Tyrosine phosphorylation of Pyk2 and downstream activation of ERK were blocked by general tyrosine kinase inhibitors herbimycin A (Herb A) and PP2, by chelation of intracellular calcium release with EGTA/BAPTA, and by inhibition of PKC by Ro-31-8220. Addition of cytoskeletal inhibitors cytochalasin-D (cyto-D), latrunculin-B (lat-B), and RGDS tetrapeptides, which disrupt focal adhesions, confirmed the association of Pyk2 tyrosine phosphorylation with ERK activation. GnRH induced a focal adhesion-anchored signaling complex between c-Src and Pyk2, because Herb A, cyto-D, and lat-B all blocked the association. Phosphorylated Pyk2 bound Grb2, and this association of Pyk2 activity, signaling complex formation, and ERK activation was confirmed using point mutations of Pyk2. Expression of a c-Src activation site mutant (Y402F) and mutation of the Grb2 binding site (Y881F) in Pyk2 all abrogated activation of ERK. Using dominant-negative mutants and the MEK1/2 inhibitor PD98059, we identified that the signaling complex activated Ras, c-Raf, and MEK, which induced nuclear localization of ERK. The nuclear focus of this Pyk2-dependent ERK-activated pathway was Egr-1. Using PD98059, constitutively active Egr-1, DNA resections, and point mutation of Egr-1 DNA binding sites within the LH β -subunit gene promoter mapped ERK-responsive Egr-1 dependence to the PP and not the upstream response region (URR). *Broken lines* indicate activation pathways elucidated by others (5,13,14,58, 63), whereas *solid lines* indicate direct activation demonstrated in this study and are described above. *Light blue boxes* are labeled with the inhibitor and dominant negative mutants used and are positioned at the appropriate point of the MAPK signaling cascade. PLC- β , Phospholipase C- β .

Table 1
Synthetic Oligonucleotides Used for Egr-1 Pull-Down and PCR Experiments

Name	Position (bp)	DNA Sequence (5'–3')
pEgr-1	–55/–32	TGGCCTTGCCGCCCCC <u>ACAGCCTG</u>
mpEgr-1	–55/–32	TGGCCTTGCCGCCCC TTAG AGCCTG ^a
dEgr-1	–114/–91	GCCTCTCGCCCCCGGGGAGATTAG
mdEgr-1	–114/–91	GCCTCT AGTACT CGGGGAGATTAG ^a
oS2	–126/–100	CTGACCTTGTCTGCCTCTCGCCCCCGG
omS2	–126/–100	CTGACCTTGTCTGCCTCT AGTACT CG ^a
oAS2	–46/–22	CTTATACCTGCAGGCTGTGGGGGCG
omAS2	–46/–22	CTTATACCTGCAGGCT CTAAGGGCG ^a
oCS2	–35/–16	CCTGCAGGTATAAGACTAGG
oACS2	+19/pA3Luc	CGGAATGCC AAGCTT AATTTCG ^b
oS1	–692/–675	GGGGTACC AGATCTTGCCCTTGGAC ^b
oAS1	–20/+13	CCCCAACTTGCTCCCTGCTGTGTTACCTAG
Egr-1ex1S	–7/+14	GAGTCGCGAGAGATCCAGCCG
Egr-1ex1ASFLAG	+572/+587	CTTGTCATCGTCGTCCTTG TAGTCTGCGGTCAGGTGCTCG ^c
Egr-1ex2SFLAG	+1274/+1292	GACTACAAGGACGACGATGACAAGG AGTCTTTTCCTGACATCTC ^c
Egr-1ex2AS	+2665/+2385	CTCCTCTCCTATGGCCATCTC

Nucleotide sequence numbering in base pairs (bp) is relative to the transcription start site of the ovine LH β -subunit gene or the human Egr-1 gene. PCR primers are shown as sense (S) or antisense (AS). Putative Egr-1 DNA binding sites are *underlined*.

are highlighted in *bold*.

^c FLAG tag

^b *Hind*III and *Kpn*I restriction enzyme sites

^a point mutations in pEgr-1 and dEgr-1 DNA binding site